

NON-TRANSGENIC HERBICIDE RESISTANT PLANTS

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NON-TRANSGENIC HERBICIDE RESISTANT PLANTS

The present application claims priority to U.S. Provisional Application No. 60/158,027, filed on October 7, 1999 and to U.S. Provisional Application No. 60/173,564, filed December 30, 1999, the disclosures of each of which are incorporated by reference herein in their entirety.

1. FIELD OF THE INVENTION

The present invention relates to the production of a non-transgenic plant resistant or tolerant to a herbicide of the phosphonomethylglycine family, *e.g.*, glyphosate. The present invention also relates to the use of a recombinogenic oligonucleobase to make a desired mutation in the chromosomal or episomal sequences of a plant in the gene encoding for 5-enol pyruvylshikimate-3-phosphate synthase (EPSPS). The mutated protein, which substantially maintains the catalytic activity of the wild-type protein, allows for increased resistance or tolerance of the plant to a herbicide of the phosphonomethylglycine family, and allows for the substantially normal growth or development of the plant, its organs, tissues or cells as compared to the wild-type plant irrespective of the presence or absence of the herbicide. The present invention also relates to a non-transgenic plant cell in which the EPSPS gene has been mutated, a non-transgenic plant regenerated therefrom, as well as a plant resulting from a cross using a regenerated non-transgenic plant having a mutated EPSPS gene.

2. BACKGROUND TO THE INVENTION

2.1 PHOSPHONOMETHYLGLYCINE HERBICIDES

Herbicide-tolerant plants may reduce the need for tillage to control weeds thereby effectively reducing soil erosion. One herbicide which is the subject of much investigation in this regard is N-phosphonomethylglycine, commonly referred to as glyphosate. Glyphosate inhibits the shikimic acid pathway which leads to the biosynthesis of aromatic compounds including amino acids, hormones and vitamins. Specifically, glyphosate curbs the conversion of phosphoenolpyruvic acid (PEP) and 3-phosphoshikimic acid to 5-enolpyruvyl-3-phosphoshikimic acid by inhibiting the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (hereinafter referred to as EPSP synthase or EPSPS). For purposes of the present invention, the term "glyphosate" includes any

e.g., glyphosate of the phosphonomethylglycine family.

Tolerance of plants to glyphosate can be increased by introducing a mutant EPSPS gene having an alteration in the EPSPS amino acid coding sequence into the genome of the plant. Examples of some of the mutations in the EPSPS gene for inducing glyphosate tolerance are described in the following patents: U.S. Patent No. 5,310,667; U.S. Patent No. 5,866,775; U.S. Patent No. 5,312,910; U.S. Patent No. 5,145,783. These proposed mutations typically have a higher K_i for glyphosate than the wild-type EPSPS enzyme which confers the glyphosate-tolerant phenotype, but these variants are also characterized by a high K_m for PEP which makes the enzyme kinetically less efficient (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663; Schulz et al., 1984, Arch. Microbiol. 137: 121-123; Sost et al., 1984, FEBS Lett. 173: 238-241; Kishore et al., 1986, Fed. Proc. 45: 1506; Sost and Amrhein, 1990, Arch. Biochem. Biophys. 282: 433-436). Many mutations of the EPSPS gene are chosen so as to produce an EPSPS enzyme that is resistant to herbicides, but unfortunately, the EPSPS enzyme produced by the mutated EPSPS gene has a significantly lower enzymatic activity than the wild-type EPSPS. For example, the apparent K_m for PEP and the apparent K_i for glyphosate for the wild-type EPSPS from *E. coli* are 10 μ M and 0.5 μ M, while for a glyphosate-tolerant isolate having a single amino acid substitution of alanine for glycine at position 96, these values are 220 μ M and 4.0 mM, respectively. A number of glyphosate-tolerant EPSPS genes have been constructed by mutagenesis. Again, the glyphosate-tolerant EPSPS had lower catalytic efficiency (V_{max}/K_m), as shown by an increase in the K_m for PEP, and a slight reduction of the V_{max} of the wild-type plant enzyme (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663).

Since the kinetic constants of the variant enzymes are impaired with respect to PEP, it has been proposed that high levels of overproduction of the variant enzyme, 40-80 fold, would be required to maintain normal catalytic activity in plants in the presence of glyphosate (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663). It has been shown that glyphosate-tolerant plants can be produced by inserting into the genome of the plant the capacity to produce a higher level of EPSP synthase in the chloroplast of the cell (Shah et al., 1986, Science 233, 478-481), which enzyme is preferably glyphosate-tolerant (Kishore et al., 1988, Ann. Rev. Biochem. 57:627-663).

The introduction of the exogenous mutant EPSPS genes into plant is well documented. For example, according to U.S. Patent No. 4,545,060, to increase a plant's resistance to glyphosate, a gene coding for an EPSPS variant having at least one mutation that renders the enzyme more resistant to its competitive inhibitor, *i.e.*, glyphosate, is

where EPSPS gene product of a mutant EPSPS gene product with significantly lower

enzymatic activity as compared to wild type. The low expression or low enzymatic activity of the mutated enzyme results in abnormally low levels of growth and development of the plant.

While such variants in the EPSP synthases have proved useful in obtaining
5 transgenic plants tolerant to glyphosate, it would be increasingly beneficial to obtain a variant EPSPS gene product that is highly glyphosate-tolerant but still kinetically efficient, such that improved tolerance can be obtained with a wild-type expression level.

2.2 RECOMBINAGENIC OLIGONUCLEOBASES

10 Recombinagenic oligonucleobases and their use to effect genetic changes in eukaryotic cells are described in United States patent No. 5,565,350 to Kmiec (Kmiec I). Kmiec I teaches a method for introducing specific genetic alterations into a target gene. Kmiec I discloses, *inter alia*, recombinagenic oligonucleobases having two strands, in which a first strand contains two segments of at least 8 RNA-like nucleotides that are
15 separated by a third segment of from 4 to about 50 DNA-like nucleotides, termed an "interposed DNA segment." The nucleotides of the first strand are base paired to DNA-like nucleotides of a second strand. The first and second strands are additionally linked by a segment of single stranded nucleotides so that the first and second strands are parts of a single oligonucleotide chain. Kmiec I further teaches a method for introducing specific
20 genetic alterations into a target gene. According to Kmiec I, the sequences of the RNA segments are selected to be homologous, *i.e.*, identical, to the sequence of a first and a second fragment of the target gene. The sequence of the interposed DNA segment is homologous with the sequence of the target gene between the first and second fragment except for a region of difference, termed the "heterologous region." The heterologous
25 region can effect an insertion or deletion, or can contain one or more bases that are mismatched with the sequence of target gene so as to effect a substitution. According to Kmiec I, the sequence of the target gene is altered as directed by the heterologous region, such that the target gene becomes homologous with the sequence of the recombinagenic oligonucleobase. Kmiec I specifically teaches that ribose and 2'-O-methylribose, *i.e.*, 2'-
30 methoxyribose, containing nucleotides can be used in recombinagenic oligonucleobases and that naturally-occurring deoxyribose-containing nucleotides can be used as DNA-like nucleotides.

U.S. Patent No. 5,731,181 to Kmiec (Kmiec II) specifically disclose the use

of recombinagenic genetic changes in specific target genes. Other patents discussing the use

of recombinagenic oligonucleobases include: U.S. Patent Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT US00 23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

- 5 Recombinagenic oligonucleobases include mixed duplex oligonucleotides, non-nucleotide containing molecules taught in Kmiec II and other molecules taught in the above-noted patents and patent publications.

Citation or identification of any reference in Section 2, or any section of this application shall not be construed as an admission that such reference is available as prior art to the present invention.

3. SUMMARY OF THE INVENTION

The present invention is directed to a non-transgenic plant or plant cell having one or more mutations in the EPSPS gene, which plant has increased resistance or tolerance to a member of the phosphonomethylglycine family and which plant exhibits substantially normal growth or development of the plant, its organs, tissues or cells, as compared to the corresponding wild-type plant or cell. The present invention is also directed to a non-transgenic plant having a mutation in the EPSPS gene, which plant is resistant to or has an increased tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate, wherein the mutated EPSPS protein has substantially the same catalytic activity as compared to the wild-type EPSPS protein.

The present invention is also directed to a method for producing a non-transgenic plant having a mutated EPSPS gene that substantially maintains the catalytic activity of the wild-type protein irrespective of the presence or absence of a herbicide of the phosphonomethylglycine family. The method comprises introducing into a plant cell a recombinagenic oligonucleobase with a targeted mutation in the EPSPS gene and identifying a cell, seed, or plant having a mutated EPSPS gene.

Illustrative examples of a recombinagenic oligonucleobase is found in following patent publications, which are incorporated in their entirety by reference herein:

30 U.S. Patent Nos. 5,565,350; 5,756,325; 5,871,984; 5,760,012; 5,731,181; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT US00 23457; and in International Patent Publication Nos. WO 98/49350; WO

35 gymnospermous plant, including *e.g.*, conifers, and angiospermous plant, including *e.g.*, herbaceous species, or any species that produces edible fruits, seeds or vegetables, or any

species that produces colorful or aromatic flowers. For example, the plant may be selected from a species of plant from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax, oilseed rape, cucumber, morning glory, balsam, pepper, eggplant, marigold, lotus, cabbage, daisy, carnation, tulip, iris, lily, and nut producing plants insofar as they are not already specifically mentioned.

The recombinagenic oligonucleobase can be introduced into a plant cell using any method commonly used in the art, including but not limited to, microcarriers (biolistic delivery), microfibers, electroporation, microinjection.

The invention is also directed to the culture of cells mutated according to the methods of the present invention in order to obtain a plant that produces seeds, henceforth a "fertile plant", and the production of seeds and additional plants from such a fertile plant.

The invention is further directed to a method of selectively controlling weeds in a field, the field comprising plants with the disclosed EPSPS gene alterations and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

The invention is also directed to novel mutations in the EPSPS gene that confer resistance or tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate, to a plant or wherein the mutated EPSPS has substantially the same enzymatic activity as compared to wild-type EPSPS.

3.1 DEFINITIONS

The invention is to be understood in accordance with the following definitions.

An oligonucleobase is a polymer of nucleobases, which polymer can hybridize by Watson-Crick base pairing to a DNA having the complementary sequence.

Nucleobases comprise a base, which is a purine, pyrimidine, or a derivative or analog thereof. Nucleobases include peptide nucleobases, the subunits of peptide nucleic acids, and morpholine nucleobases as well as nucleosides and nucleotides. Nucleosides are nucleobases that contain a pentosefuranosyl moiety, *e.g.*, an optionally substituted riboside or 2'-deoxyriboside. Nucleosides can be linked by one of several linkage moieties, which may or may not contain a phosphorus. Nucleosides that are linked by unsubstituted phosphodiester linkages are termed nucleotides.

The nucleobase chain can contain

nucleobases of all types. An oligonucleobase compound is a compound comprising one or more oligonucleobase chains that are complementary and hybridized by Watson-Crick base pairing. Nucleobases are either deoxyribo-type or ribo-type. Ribo-type nucleobases are pentosefuranosyl containing nucleobases wherein the 2' carbon is a methylene substituted with a hydroxyl, alkyloxy or halogen. Deoxyribo-type nucleobases are nucleobases other than ribo-type nucleobases and include all nucleobases that do not contain a pentosefuranosyl moiety.

An oligonucleobase strand generically includes both oligonucleobase chains and segments or regions of oligonucleobase chains. An oligonucleobase strand has a 3' end and a 5' end. When a oligonucleobase strand is coextensive with a chain, the 3' and 5' ends of the strand are also 3' and 5' termini of the chain.

According to the present invention, substantially normal growth of a plant, plant organ, plant tissue or plant cell is defined as a growth rate or rate of cell division of the plant, plant organ, plant tissue, or plant cell that is at least 35%, at least 50%, at least 60%, or at least 75% of the growth rate or rate of cell division in a corresponding plant, plant organ, plant tissue or plant cell expressing the wild type EPSPS protein.

According to the present invention, substantially normal development of a plant, plant organ, plant tissue or plant cell is defined as the occurrence of one or more developmental events in the plant, plant organ, plant tissue or plant cell that are substantially the same as those occurring in a corresponding plant, plant organ, plant tissue or plant cell expressing the wild type EPSPS protein.

According to the present invention plant organs include, but are not limited to, leaves, stems, roots, vegetative buds, floral buds, meristems, embryos, cotyledons, endosperm, sepals, petals, pistils, carpels, stamens, anthers, microspores, pollen, pollen tubes, ovules, ovaries and fruits, or sections, slices or discs taken therefrom. Plant tissues include, but are not limited to, callus tissues, ground tissues, vascular tissues, storage tissues, meristematic tissues, leaf tissues, shoot tissues, root tissues, gall tissues, plant tumor tissues, and reproductive tissues. Plant cells include, but are not limited to, isolated cells with cell walls, variously sized aggregates thereof, and protoplasts.

Plants are substantially "tolerant" to glyphosate when they are subjected to it and provide a dose-response curve which is shifted to the right when compared with that provided by similarly subjected non-tolerant like plant. Such dose response curves have "dose" plotted on the X-axis and "percentage kill", "herbicidal effect", etc., plotted on the

Y-axis. Such tolerance may be exhibited by a whole plant or other regions, when subjected to

glyphosate at concentrations and rates which are typically employed by the agrochemical community to kill weeds in the field. Plants which are resistant to a herbicide are also tolerant of the herbicide. The terms "resistant" and "tolerant" are to be construed as "tolerant and/or resistant" within the context of the present application.

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4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1A is the DNA sequence of *Arabidopsis thaliana* EPSPS gene (SEQ ID NO:1). The bold underlined nucleotide residues are the targeted residues.

FIG. 1B is the amino acid sequence of *Arabidopsis thaliana* EPSPS protein
10 (SEQ ID NO:2). The bold and underlined amino acid residues are the targeted residues.

FIG. 2 is a list of the *Arabidopsis thaliana* wild-type and mutant EPSPS nucleotide and amino acid sequences in the region of amino acid position 173 to 183; wild-type nucleotide sequence (SEQ ID NO:1) and wild-type amino acid sequence (SEQ ID NO:2); mutant A₁₇₇ nucleotide sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4); mutant I₁₇₈ nucleotide sequence (SEQ ID NO:5) and amino acid sequence (SEQ ID NO:6); mutant A₁₇₇I₁₇₈ nucleotide sequence (SEQ ID NO:7) and amino acid sequence (SEQ ID NO:8); mutant I₁₇₈S₁₈₂ nucleotide sequence (SEQ ID NO:9) and amino acid sequence (SEQ ID NO:10); mutant A₁₇₇S₁₈₂ nucleotide sequence (SEQ ID NO:11) and amino acid sequence (SEQ ID NO:12); mutant A₁₇₇I₁₇₈S₁₈₂ nucleotide sequence (SEQ ID NO:13) and
20 amino acid sequence (SEQ ID NO:14); mutant V₁₇₇S₁₈₂ nucleotide sequence (SEQ ID NO:15) and amino acid sequence (SEQ ID NO:16); mutant L₁₇₈S₁₈₂ nucleotide sequence (SEQ ID NO:17) and amino acid sequence (SEQ ID NO:18); mutant A₁₇₇V₁₇₈ nucleotide sequence (SEQ ID NO:19) and amino acid sequence (SEQ ID NO:20); mutant A₁₇₇L₁₈₂ nucleotide sequence (SEQ ID NO:21) and amino acid sequence (SEQ ID NO:22).

FIG. 3A-C is an alignment of the DNA of *Arabidopsis thaliana* EPSPS gene performed by DNASTar (LaserGene), (SEQ ID NO:1) with the nucleotide sequences of *Brassica napus* (SEQ ID NO:23); *Petunia hybrida* (SEQ ID NO:24); and *Zea mays* (SEQ ID NO:25) EPSPS gene. The sequences are aligned using J. Hein method with weighted residue weight table.

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FIG. 4 is an alignment of the *Arabidopsis thaliana* EPSPS amino acid sequence (SEQ ID NO:2) with the *Brassica napus* (SEQ ID NO:26); *Petunia hybrida* (SEQ ID NO:27); and *Zea mays* (SEQ ID NO:28) EPSPS amino acid sequences. The sequences

are aligned using J. Hein method with weighted residue weight table. The sequences are: *Arabidopsis thaliana* (SEQ ID NO:2); mutant primer 1 (SEQ ID NO:59);

mutant primer A₁--I₁₋₈ (SEQ ID NO:31); mutant primer I₁₋₈S₁₈₂ (SEQ ID NO:32); mutant primer A₁--S₁₈₂ (SEQ ID NO:34); mutant primer A₁--I₁₋₈S₁₈₂ (SEQ ID NO:35); mutant primer V₁--S₁₈₂ (SEQ ID NO:35); mutant primer L₁₋₈S₁₈₂ (SEQ ID NO:36); mutant primer A₁--V₁₋₈ (SEQ ID NO:37); and mutant primer A₁--L₁₈₂ (SEQ ID NO:38)).

5 FIG. 6 is the growth measured by optical density at 600 nm of *Arabidopsis* clones in the presence (+) and absence (-) of 17 mM glyphosate.

FIG. 7 (top panel) is a western blot showing the expression of His-tagged *Bacillus*, *Arabidopsis* wild type (WT) and mutant (AS) EPSPS proteins isolated from cell lysates (L) and eluates (E). Untransformed *Salmonella* as a negative control shows no
10 EPSPS expression. The bottom panel is a silver-stained duplicate gel.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a non-transgenic plant or plant cell having a mutation in the EPSPS gene, which plant has increased resistance or tolerance to a
15 member of the phosphonomethylglycine family and which plant exhibits substantially normal growth or development of the plant, its organs, tissues or cells, as compared to the corresponding wild-type plant or cell. The present invention is also directed to a non-transgenic plant having a mutation in the EPSPS gene, which plant is resistant to or has an increased tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate.
20 wherein the mutated EPSPS protein has substantially the same catalytic activity as compared to the wild-type EPSPS protein.

The present invention is also directed to a method for producing a non-transgenic plant having a mutated EPSPS gene that substantially maintains the catalytic activity of the wild-type protein irrespective of the presence or absence of a herbicide of the
25 phosphonomethylglycine family. The method comprises introducing into a plant cell a recombinagenic oligonucleobase with a targeted mutation in the EPSPS gene and identifying a cell, seed, or plant having a mutated EPSPS gene.

Illustrative examples of a recombinagenic oligonucleobase is found in following patent publications, which are incorporated in their entirety by reference herein:
30 U.S. Patent Nos. 5,565,350; 5,756,325; 5,871,984; 5,760,012; 5,731,181; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Patent No. PCT US00 23457; and in International Patent Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789.

characterized species, or any species that produces edible fruits, seeds or vegetables, or any

species that produces colorful or aromatic flowers. For example, the plant may be selected from a species of plant from the group consisting of canola, sunflower, tobacco, sugar beet, cotton, maize, wheat, barley, rice, sorghum, tomato, mango, peach, apple, pear, strawberry, banana, melon, potato, carrot, lettuce, onion, soya spp, sugar cane, pea, field beans, poplar, grape, citrus, alfalfa, rye, oats, turf and forage grasses, flax, oilseed rape, cucumber, morning glory, balsam, pepper, eggplant, marigold, lotus, cabbage, daisy, carnation, tulip, iris, lily, and nut producing plants insofar as they are not already specifically mentioned.

The recombinagenic oligonucleobase can be introduced into a plant cell using any method commonly used in the art, including but not limited to, microcarriers (biolistic delivery), microfibers, electroporation, microinjection.

The invention is also directed to the culture of cells mutated according to the methods of the present invention in order to obtain a plant that produces seeds, henceforth a "fertile plant", and the production of seeds and additional plants from such a fertile plant.

The invention is further directed to a method of selectively controlling weeds in a field, the field comprising plants with the disclosed EPSPS gene alterations and weeds, the method comprising application to the field of a herbicide to which the said plants have been rendered resistant.

The invention is also directed to novel mutations in the EPSPS gene that confer resistance or tolerance to a member of the phosphonomethylglycine family, *e.g.*, glyphosate, to a plant or wherein the mutated EPSPS has substantially the same enzymatic activity as compared to wild-type EPSPS.

5.1 RECOMBINAGENIC OLIGONUCLEOBASES

The invention can be practiced with recombinagenic oligonucleobases having the conformations and chemistries described in United States patent No. 5,565,350 to Kmiec (Kmiec I) and U.S. patent No. 5,731,181 (Kmiec II) gene, which are hereby incorporated by reference. Kmiec I teaches a method for introducing specific genetic alterations into a target gene. The recombinagenic oligonucleobases in Kmiec I and or Kmiec II contain two complementary strands, one of which contains at least one segment of RNA-type nucleotides (an "RNA segment") that are base paired to DNA-type nucleotides of the other strand.

Kmiec II discloses that purine and pyrimidine base-containing non-nucleotides can be substituted for nucleotides. U.S. Patent Nos. 5,756,325; 5,871,984;

invention can be practiced with mixed duplex oligonucleotides having two or more types of RNA-type nucleotides. The function of an RNA segment may not be affected by an interruption caused by the introduction of a deoxynucleotide between two RNA-type trinucleotides, accordingly, the term RNA segment encompasses such an "interrupted RNA segment." An uninterrupted RNA segment is termed a contiguous RNA segment. In an alternative embodiment an RNA segment can contain alternating RNase-resistant and unsubstituted 2'-OH nucleotides. The mixed duplex oligonucleotides preferably have fewer than 100 nucleotides and more preferably fewer than 85 nucleotides, but more than 50 nucleotides. The first and second strands are Watson-Crick base paired. In one embodiment the strands of the mixed duplex oligonucleotide are covalently bonded by a linker, such as a single stranded hexa, penta or tetranucleotide so that the first and second strands are segments of a single oligonucleotide chain having a single 3' and a single 5' end. The 3' and 5' ends can be protected by the addition of a "hairpin cap" whereby the 3' and 5' terminal nucleotides are Watson-Crick paired to adjacent nucleotides. A second hairpin cap can, additionally, be placed at the junction between the first and second strands distant from the 3' and 5' ends, so that the Watson-Crick pairing between the first and second strands is stabilized.

The first and second strands contain two regions that are homologous with two fragments of the target EPSPS gene, *i.e.*, have the same sequence as the target gene. A homologous region contains the nucleotides of an RNA segment and may contain one or more DNA-type nucleotides of connecting DNA segment and may also contain DNA-type nucleotides that are not within the intervening DNA segment. The two regions of homology are separated by, and each is adjacent to, a region having a sequence that differs from the sequence of the target gene, termed a "heterologous region." The heterologous region can contain one, two or three mismatched nucleotides. The mismatched nucleotides can be contiguous or alternatively can be separated by one or two nucleotides that are homologous with the target gene. Alternatively, the heterologous region can also contain an insertion or one, two, three or of five or fewer nucleotides. Alternatively, the sequence of the mixed duplex oligonucleotide may differ from the sequence of the target gene only by the deletion of one, two, three, or five or fewer nucleotides from the mixed duplex oligonucleotide. The length and position of the heterologous region is, in this case, deemed to be the length of the deletion, even though no nucleotides of the mixed duplex oligonucleotide are within the heterologous region. The distance between the fragments of

Figure 1 is a schematic diagram of a mixed duplex oligonucleotide. The homologous regions are thereby separated by

the mixed duplex oligonucleotide farther than their complementary homologous fragments are in the gene, and the converse is applicable when the heterologous region encodes a deletion.

The RNA segments of the mixed duplex oligonucleotides are each a part of a
5 homologous region, *i.e.*, a region that is identical in sequence to a fragment of the target gene, which segments together preferably contain at least 13 RNA-type nucleotides and preferably from 16 to 25 RNA-type nucleotides or yet more preferably 18-22 RNA-type nucleotides or most preferably 20 nucleotides. In one embodiment, RNA segments of the homology regions are separated by and adjacent to, *i.e.*, "connected by" an intervening
10 DNA segment. In one embodiment, each nucleotide of the heterologous region is a nucleotide of the intervening DNA segment. An intervening DNA segment that contains the heterologous region of a mixed duplex oligonucleotide is termed a "mutator segment."

The change to be introduced into the target EPSPS gene is encoded by the heterologous region. The change to be introduced into the EPSPS gene may be a change in
15 one or more bases of the EPSPS gene sequence or the addition or deletion of one or more bases.

In another embodiment of the present invention, the recombinagenic oligonucleobase is a single stranded oligodeoxynucleotide mutational vector or SSOMV, which is disclosed in International Patent Application PCT/US00/23457, which is
20 incorporated by reference in its entirety. The sequence of the SSOMV is based on the same principles as the mutational vectors described in U.S. Patent Nos. 5,756,325; 5,871,984; 5,760,012; 5,888,983; 5,795,972; 5,780,296; 5,945,339; 6,004,804; and 6,010,907 and in International Publication Nos. WO 98/49350; WO 99/07865; WO 99/58723; WO 99/58702; and WO 99/40789. The sequence of the SSOMV contains two regions that are homologous
25 with the target sequence separated by a region that contains the desired genetic alteration termed the mutator region. The mutator region can have a sequence that is the same length as the sequence that separates the homologous regions in the target sequence, but having a different sequence. Such a mutator region can cause a substitution. Alternatively, the homologous regions in the SSOMV can be contiguous to each other, while the regions in the
30 target gene having the same sequence are separated by one, two or more nucleotides. Such a SSOMV causes a deletion from the target gene of the nucleotides that are absent from the SSOMV. Lastly, the sequence of the target gene that is identical to the homologous regions may be adjacent in the target gene but separated by one two or more nucleotides in

The nucleotides of the SSOMV are deoxyribonucleotides that are linked by unmodified phosphodiester bonds except that the 3' terminal and/or 5' terminal internucleotide linkage or alternatively the two 3' terminal and/or 5' terminal internucleotide linkages can be a phosphorothioate or phosphoamidate. As used herein an internucleotide linkage is the linkage between nucleotides of the SSOMV and does not include the linkage between the 3' end nucleotide or 5' end nucleotide and a blocking substituent, see *supra*. In a specific embodiment the length of the SSOMV is between 21 and 55 deoxynucleotides and the lengths of the homology regions are, accordingly, a total length of at least 20 deoxynucleotides and at least two homology regions should each have lengths of at least 8 deoxynucleotides.

The SSOMV can be designed to be complementary to either the coding or the non-coding strand of the target gene. When the desired mutation is a substitution of a single base, it is preferred that both the mutator nucleotide be a pyrimidine. To the extent that is consistent with achieving the desired functional result it is preferred that both the mutator nucleotide and the targeted nucleotide in the complementary strand be pyrimidines. Particularly preferred are SSOMV that encode transversion mutations, *i.e.*, a C or T mutator nucleotide is mismatched, respectively, with a C or T nucleotide in the complementary strand.

In addition to the oligodeoxynucleotide the SSOMV can contain a 5' blocking substituent that is attached to the 5' terminal carbons through a linker. The chemistry of the linker is not critical other than its length, which should preferably be at least 6 atoms long and that the linker should be flexible. A variety of non-toxic substituents such as biotin, cholesterol or other steroids or a non-intercalating cationic fluorescent dye can be used. Particularly preferred as reagents to make SSOMV are the reagents sold as Cy3TM and Cy5TM by Glen Research, Sterling VA, which are blocked phosphoroamidites that upon incorporation into an oligonucleotide yield 3,3,3',3'-tetramethyl N,N'-isopropyl substituted indomonocarboyanine and indodicarboyanine dyes, respectively. Cy3 is the most preferred. When the indocarboyanine is N-oxyalkyl substituted it can be conveniently linked to the 5' terminal of the oligodeoxynucleotide through as a phosphodiester with a 5' terminal phosphate. The chemistry of the dye linker between the dye and the oligodeoxynucleotide is not critical and is chosen for synthetic convenience. When the commercially available Cy3 phosphoramidite is used as directed the resulting 5' modification consists of a blocking substituent and linker together which are a

5' blocking substituent and linker together which are a

contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

5 Alternatively, and/or additionally, the mutation may result in the replacement of any amino acid at positions corresponding to 256, 284-288 and 353-356 with respect to the EPSPS protein depicted in Figure 1B (SEQ ID NO. 2).

 In specific embodiments of the present invention, the EPSPS gene is mutated at amino acid position 177 in which Gly is replaced by Ala. Another specific embodiment
10 is the substitution of Thr at amino acid position 178 by Ile. A further specific embodiment comprises a mutation at amino acid position 177 in which Gly is replaced by Ala, plus the additional substitution of Thr at amino acid position 178 by Ile. Other specific
embodiments of the present invention are directed to mutations at amino acid position 178, in which Thr is replaced by Ile, plus the additional mutation at position 182, in which Pro is
15 replaced by Ser. Other embodiments include the substitution of Gly at amino acid position 177 by Ala, plus the additional mutation at amino acid position 182, in which Pro is substituted by Ser. Other mutated EPSPS sequences comprise the substitution of Gly at
position 177 by Ala, plus the substitution at position 178, in which Thr is replaced by Ile, plus the additional substitution of Pro at amino acid position 182 by Ser. Another
20 embodiment is the substitution of Thr at amino acid position 178 by Val and the additional mutation at amino acid position 182, in which Pro is replaced by Ser. A further specific
embodiment includes the substitution of Thr at position 178 by Leu, plus the mutation at amino acid position 182, in which Pro is replaced by Ser. A further embodiment includes,
the substitution at amino acid position 177 in which Gly is replaced by Ala, plus the
25 substitution of Thr at position 178 by Val. The invention also embodies the substitution at amino acid position 177 in which Gly is replaced by Ala, plus the replacement of Thr at
amino acid position 178 by Leu (see Figure 2).

 The foregoing mutations in the EPSPS gene were described using the *Arabidopsis thaliana* EPSPS gene (SEQ ID NO:1) and protein (SEQ ID NO:2). The
30 present invention also encompasses mutant EPSPS genes of other species (paralogs). However, due to variations in the EPSPS genes of different species, the number of the amino acid residue to be changed in one species may be different in another species. Nevertheless, the analogous position is readily identified by one of skill in the art by

comparing the amino acid sequence of the EPSPS gene of *Arabidopsis thaliana* with the amino acid sequence of the EPSPS gene of other species. Thus, the analogous

positions in *Zea mays* are Leu₉₇, Gly₁₀₁, Thr₁₀₂, Ala₁₀₃, Met₁₀₄, Arg₁₀₅, Pro₁₀₆, Ser₂₃, Ser₁₇₉ and Leu₁₂₂. Thus, the *Zea mays* EPSPS amino acid sequence is mutated at one or more of the following amino acid positions and results in one or more of the following substitutions:

- (i) Leu₉₇ - Phe
- 5 (ii) Gly₁₀₁ - Ala or Ile
- (iii) Thr₁₀₂ - Ile or Val or Leu
- (iv) Ala₁₀₃ - Gly
- (v) Met₁₀₄ - Cys
- (vi) Arg₁₀₅ - Leu or Ser
- 10 (vii) Pro₁₀₆ - Leu or Ser
- (viii) Ser₂₃ -Asp
- (ix) Ser₁₇₉ -Ala
- (x) Leu₁₂₂ -Lys.

In another embodiment of the present invention, within the *Zea mays* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Phe-Leu-Gly-Asn (SEQ ID NO:39) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

In *Brassica napus*, the analogous amino acid positions are Leu₁₆₉, Gly₁₇₃, Thr₁₇₄, Ala₁₇₅, Met₁₇₆, Arg₁₇₇, Pro₁₇₈, Ser₉₄, Ser₇₅₁ and Leu₁₀₄. Thus, the *Brassica napus*

- (ii) Gly₁₇₃ - Ala or Ile
- (iii) Thr₁₇₄ - Ile or Val or Leu
- (iv) Ala₁₇₅ - Gly
- (v) Met₁₇₆ - Cys
- (vi) Arg₁₇₇ - Leu or Ser
- (vii) Pro₁₇₈ - Leu or Ser
- (viii) Ser₉₄ - Asp
- (ix) Ser₂₅₁ - Ala
- (x) Leu₁₉₄ - Lys

10 In another embodiment of the present invention, within the *Brassica napus* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Tyr-Leu-Gly-Asn (SEQ ID NO:29) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

In *Petunia hybrida* the analogous positions are Leu₁₆₉, Gly₁₇₃, Thr₁₇₄, Ala₁₇₅, Met₁₇₆, Arg₁₇₇, Pro₁₇₈, Ser₉₄, Ser₂₅₁ and Leu₁₉₄. Thus, the *Petunia hybrida* EPSPS amino acid sequence is mutated at one or more of the following amino acid positions and results in one or more of the following substitutions:

- (i) Leu₁₆₉ - Phe
- (ii) Gly₁₇₃ - Ala or Ile

(vi) Arg₁₇₇ - Leu or Ser

(vii) Pro₁₇₈ - Leu or Ser

(viii) Ser₉₄ -Asp

(ix) Ser₂₅₁ -Ala

5 (x) Leu₁₀₄ -Lys

In another embodiment of the present invention, within the *Petunia hybrida* EPSPS gene product the amino acid residue to be changed is Leu within the contiguous sequence Leu-Phe-Leu-Gly-Asn (SEQ ID NO:39) and is changed to Phe; or the amino acid residue to be changed is Gly within the contiguous sequence Asn-Ala-Gly-Thr-Ala (SEQ ID NO:30) and is changed to Ala or Ile; or the amino acid to be changed is Thr within the contiguous sequence Ala-Gly-Thr-Ala-Met (SEQ ID NO:31) and is changed to Ile, Val or Leu; or the amino acid to be changed is Ala within the contiguous sequence Gly-Thr-Ala-Met-Arg (SEQ ID NO:32) and is changed to Gly; or the amino acid to be changed is Met within the contiguous sequence Thr-Ala-Met-Arg-Pro (SEQ ID NO:33) and is changed to Cys; or the amino acid to be changed is Arg within the contiguous sequence Ala-Met-Arg-Pro-Leu (SEQ ID NO:34) and is changed to Leu or Ser; or the amino acid to be changed is Pro within the contiguous sequence Met-Arg-Pro-Leu-Thr (SEQ ID NO:35) and is changed to Leu or Ser; or the amino acid to be changed is Ser within a contiguous Pro-Gly-Ser-Lys-Ser (SEQ ID NO:36) and is changed to Asp; or the amino acid to be changed is Ser within the contiguous sequence Ile-Ser-Ser-Gln-Tyr (SEQ ID NO:37) and is changed to Ala; or the amino acid to be changed is Leu within the contiguous sequence Tyr-Val-Leu-Asp-Gly (SEQ ID NO:38) and is changed to Lys. In other embodiments, one or more of the foregoing changes can be made in the EPSPS amino acid sequence.

25 5.3 THE DELIVERY OF RECOMBINAGENIC OLIGONUCLEOBASES INTO PLANT CELLS

Any commonly known method can be used in the methods of the present invention to transform a plant cell with a recombinagenic oligonucleobases. Illustrative methods are listed below.

30

5.3.1 MICROCARRIERS AND MICROFIBERS

The use of metallic microcarriers (microspheres) for introducing large fragments of DNA into plant cells having cellulose cell walls by projectile penetration to microcarriers and devices for projecting them.

Specific conditions for using microcarriers in the methods of the present invention are described in International Publication WO 99/07865. In an illustrative technique, ice cold microcarriers (60 mg/ml), mixed duplex oligonucleotide (60 mg/ml) 2.5 M CaCl_2 and 0.1 M spermidine are added in that order; the mixture gently agitated, *e.g.*, by vortexing, for 10 minutes and let stand at room temperature for 10 minutes, whereupon the microcarriers are diluted in 5 volumes of ethanol, centrifuged and resuspended in 100% ethanol. Good results can be obtained with a concentration in the adhering solution of 8-10 $\mu\text{g}/\mu\text{l}$ microcarriers, 14-17 $\mu\text{g}/\text{ml}$ mixed duplex oligonucleotide, 1.1-1.4 M CaCl_2 and 18-22 mM spermidine. Optimal results were observed under the conditions of 8 $\mu\text{g}/\mu\text{l}$ microcarriers, 16.5 $\mu\text{g}/\text{ml}$ mixed duplex oligonucleotide, 1.3 M CaCl_2 and 21 mM spermidine.

Recombinagenic oligonucleobases can also be introduced into plant cells for the practice of the present invention using microfibers to penetrate the cell wall and cell membrane. U.S. Patent No. 5,302,523 to Coffee et al. describes the use of 30 x 0.5 μm and 10 x 0.3 μm silicon carbide fibers to facilitate transformation of suspension maize cultures of Black Mexican Sweet. Any mechanical technique that can be used to introduce DNA for transformation of a plant cell using microfibers can be used to deliver recombinagenic oligonucleobases for transmutation.

An illustrative technique for microfiber delivery of a recombinagenic oligonucleobase is as follows: Sterile microfibers (2 μg) are suspended in 150 μl of plant culture medium containing about 10 μg of a mixed duplex oligonucleotide. A suspension culture is allowed to settle and equal volumes of packed cells and the sterile fiber/nucleotide suspension are vortexed for 10 minutes and plated. Selective media are applied immediately or with a delay of up to about 120 hours as is appropriate for the particular trait.

5.3.2 PROTOPLAST ELECTROPORATION

In an alternative embodiment, the recombinagenic oligonucleobases can be delivered to the plant cell by electroporation of a protoplast derived from a plant part. The protoplasts are formed by enzymatic treatment of a plant part, particularly a leaf, according to techniques well known to those skilled in the art. *See, e.g.*, Gallois et al., 1996, in *Methods in Molecular Biology* 55:89-107, Humana Press, Totowa, NJ; Kipp et al., 1999, in *Methods in Molecular Biology* 133:213-221, Humana Press, Totowa, NJ. The protoplasts

5.3.3 WHISKERS AND MICROINJECTION

In yet another alternative embodiment, the recombinagenic oligonucleobase can be delivered to the plant cell by whiskers or microinjection of the plant cell. The so called whiskers technique is performed essentially as described in Frame et al., 1994, Plant J. 6:941-948. The recombinagenic oligonucleobase is added to the whiskers and used to transform the plant cells. The recombinagenic oligonucleobase may be co-incubated with plasmids comprising sequences encoding proteins capable of forming recombinase complexes in plant cells such that recombination is catalyzed between the oligonucleotide and the target sequence in the EPSPS gene.

10

5.4 SELECTION OF GLYPHOSATE RESISTANT PLANTS

Plants or plant cells can be tested for resistance or tolerance to a herbicide using commonly known methods in the art, e.g., by growing the plant or plant cell in the presence of a herbicide and measuring the rate of growth as compared to the growth rate in the absence of the herbicide.

15

6. EXAMPLE

The following experiments demonstrate the production of mutant *Arabidopsis thaliana* EPSPS genes which are resistant to the herbicide glyphosate and which allows the plant cells to maintain a growth rate

20

6.1 MATERIAL AND METHODS

6.1.1 ISOLATION OF ARABIDOPSIS THALIANA EPSPS cDNA

A 1.3 kb DNA fragment was amplified by PCR from an *Arabidopsis* cDNA library using the primers AtEXPEXPM1 and AtEXPEXP2CM-2. The two primers were designed to amplify the cDNA from the mature peptide to the termination codon. The 5' primer AtEXPEXPM1 contains an XbaI site (underlined) and the 3' primer AtEXPEXP2CM-2 contains a BglII site (underlined), sites which will be of use for cloning of the fragment into the expression vector.

30

AtEXPEXPM1

5'-GCTCTAGAGAAAGCGTCGGAGATTGTACTT-3' (SEQ ID NO:40)

flanking primer (5'ATEPS-198: 5'- GAAAGCGTCGGAGATTGTAC-3' (SEQ ID NO:44)) and one of the mutation-carrying primers (see Figure 5).

The 353bp PCR fragments obtained were purified (Qiagen PCR Purification kit) and their sequence confirmed. The fragments were then digested with PstI (underlined in the primer sequences) and BamHI and ligated to the pAtEPS-12 vector, which had itself been previously digested with PstI and BamHI. JM109 (Promega) competent cells were used for the transformation and plated onto chloramphenicol-containing LB plates. Clones from each mutagenesis experiment were then isolated and their sequence confirmed.

10 6.1.5 GLYPHOSATE RESISTANCE ASSAYS

Electrocompetent cells of SA4247, a LacZ - *Salmonella typhi* strain, were prepared according to well known procedures (see Current Protocols in Molecular Biology, (Wiley and Sons, Inc.)). 30 μ l of SA4247 competent cells were electroporated with 20 ng of each plasmid DNA encoding *Arabidopsis* wild-type and mutant EPSPS proteins, *Bacillus* 15 wild-type EPSPS, along with a mock transfection as a control. The settings for electroporation were 25 μ F, 2.5KV and 200 ohms. After electroporation, the cells were transferred into 15 mls culture tube and supplemented with 970 μ l of SOC medium. The cultures were incubated for 1 1/2 hours at 37°C at 225 rpm. 50 μ l of each culture were plated onto LB plates containing 17 μ g/ml chloramphenicol (in duplicates) and incubated 20 overnight at 37°C. On the following day, 5 colonies of each plate were picked and transferred onto M9 plates and incubated overnight at 37°C.

Colonies from the overnight incubation on solid M9 were inoculated into 4 ml of liquid M9 medium and grown overnight at 37°C. On the following day, 25 ml of liquid M9 medium containing chloramphenicol, IPTG and 17 mM or 0 mM Glyphosate 25 (Aldrich, 33775-7) were inoculated with 1-2 mls of each overnight culture (in duplicates), the starting OD (at 600 nm) was measured and all the cultures were normalized to start at the same OD. An OD measurement was taken every hour for seven hours. As a control of the bacterial growth, a culture of untransformed *Salmonella* was also inoculated into plain LB medium. In two independent experiments, the clones A₁-I₁-, A₁-V₁-, A₁-L₁-, and I₁- 30 did not grow in M9 medium, therefore the glyphosate-resistance assays could not be performed on them.

6.1.7 ISOLATION AND PURIFICATION OF THE UNDIFFERENTIATED BACTERIAL CELLS

inoculated into 100 ml of liquid LB medium containing chloramphenicol. The cells were

allowed to grow at 37 °C until they reached an OD of 0.5-0.7 (approximately 3 1/2 hours). IPTG was then added to the cultures to a concentration of 1.0 mM. The cells were grown five additional hours. They were then pelleted at 4000 rpm for 20 minutes at 4 °C.

The isolation and the purification of the His-tagged proteins were performed following the Qiagen Ni-NTA Protein Purification System. Cell lysates and eluates were run in duplicates on 12.5% acrylamide gels. One of the gels was silver-stained for immediate visualization, the second gel was transferred onto Millipore Immobilon-P membrane, and blocked overnight in 5% milk in TBS-T. The membrane was then exposed to Anti-His primary antibody solution (Amersham Pharmacia biotech, cat# 37-4710), followed by exposure to Anti-Mouse-IgG secondary antibody solution. (NIF825, from Amersham Pharmacia biotech ECL Western blotting analysis system, cat# RPN2108). Washes and detection reactions were performed according to the manufacturer instructions. Autoradiograms were developed after 5 minutes exposure.

15 6.2 RESULTS

Cells containing a mutation in the EPSPS gene produced cells that were both resistant to the herbicide glyphosate and that had a substantially similar growth rate in the absence or presence of glyphosate, as compared to the wild-type cells, irrespective of the presence of glyphosate (see Figure 6).

20 It was also demonstrated that the *Arabidopsis* clones containing a mutant EPSPS gene expressed the mutant protein at substantially the same level as the wild-type protein (see Figure 7).

The invention claimed and described herein is not to be limited in scope by the specific embodiments, including but not limited to the deposited microorganism
25 embodiments, herein disclosed since these embodiments are intended as illustrations of several aspects of the invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

30 A number of references are cited herein, the entire disclosures of which are incorporated herein, in their entirety, by reference.